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EXHIBIT 8

D.B. OLIVER

in *ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM : CELLULAR AND MOLECULAR BIOLOGY* EDS. F.C. NEIDHART ET AL
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EXHIBIT 8

6. Periplasm and Protein Secretion

DONALD B. OLIVER

Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794

INTRODUCTION

The periplasmic space lies between the inner and outer membranes of gram-negative bacteria. Because of this location, this space should not be thought of as a single homogenous compartment but rather as consisting of several distinct microenvironments created by the two boundary membranes and the peptidoglycan layer. Periplasmic proteins localized within these regions fulfill important functions in the processing of essential nutrients and their transport into the cell and in the biogenesis of the cell envelope. Periplasmic polysaccharides and other small molecules serve to buffer the cell from changing osmotic and ionic environments and thus help to preserve the more constant internal environment needed for cell growth and viability. Clearly, the periplasmic space is a dynamic structure at the crossroads between the anabolic and catabolic activities of the cell.

The purpose of this chapter is twofold: to review what is currently known about the structure and contents of the periplasmic space and to summarize our current understanding of the biogenesis of periplasmic proteins. Since the biogenesis of periplasmic proteins shares similarities with that of outer and trans-inner membrane proteins, studies of *Escherichia coli* protein secretion will be discussed collectively. For more comprehensive reading and references, see reviews on the periplasm (12, 69) and on protein secretion (16, 35, 83, 96).

STRUCTURE OF THE PERIPLASMIC SPACE

The structure of the periplasmic space of *E. coli* and *Salmonella typhimurium* has been largely inferred from electron microscopic studies. Unfortunately, the routine procedures used in specimen preparation often cause a release of the periplasmic contents and an increased separation of the inner and the outer membranes, making the periplasmic space appear enlarged and relatively empty. However, cryoelectron microscopic techniques result in an improved picture of this structure, in agreement with the better electron micrographs obtained by more conventional methods (2, 33, 34, 47, 70, 78). Thin sections reveal the cell envelope to be a multilayered structure, each layer being of a uniform thickness. The inner and outer membranes are approximately 7.5 nm thick, with each membrane having a typical double-track appearance due to the deposition of the heavy metal stain on each side. There is a 7.5-nm-thick layer next to the inside face of the outer membrane; this layer is lysozyme sensitive. In better micrographs it can be seen that the inner portion of this layer is composed of an electron-dense peptidoglycan layer approximately 2.0 nm thick. The peptidoglycan in this layer is highly cross-linked, forming the meshwork of the cell wall and thus dividing the periplasmic space into inner and outer periplasmic regions. It has been proposed that relatively un-cross-linked peptidoglycan polymers are also found in the inner periplasmic space, forming a periplasmic gel (47). The peptidoglycan layer is, in fact, attached to the outer membrane by covalent bonds with the Braun lipoprotein (20) and by strong ionic interactions with the matrix protein which forms hexagonal arrays on the peptidoglycan surface (103). These interactions explain why the peptidoglycan layer remains attached to and at a fixed distance from the outer membrane when cells are plasmolyzed. In contrast, the inner membrane shrinks away from the rest of the envelope during plasmoly-

sis, leaving a relatively empty inner periplasmic space between these two structures. However, under normal physiological conditions the inner periplasmic space is approximately 4 nm thick in cross section and has a content similar in density to that of the cytoplasm (34). The volume and hence the thickness of the inner periplasmic compartment, in fact, change somewhat depending on the osmolarity of the surrounding medium (see below).

This simple layered structure of the cell envelope is complicated by the existence of adhesion zones between the inner and outer membranes which appear to correspond to export sites for components needed for outer membrane growth. Such adhesion zones or "Bayer patches" appear as attachment sites between the inner and outer membranes when cells are plasmolyzed and examined in the electron microscope (10, 11). The extent of fusion between the two membranes is not known. There are approximately 200 to 400 such sites distributed over the membrane of actively growing cells, covering an estimated 5% of the membrane surface. Adhesion sites are not seen in cells grown to stationary phase, indicating that their appearance is growth phase dependent. Using various types of pulse-labeling techniques, it has been possible to show that adhesion sites correspond to export sites for newly synthesized polysaccharides, lipopolysaccharides, and outer membrane proteins (10, 87). Whether the export of periplasmic components also occurs at or adjacent to such sites is unknown. It appears that only a fraction (10%) of the adhesion sites may be active in the export of outer membrane components during a given period. Regulation of the formation, activity, and specificity of adhesion sites could help to explain the complex topological organization of the cell envelope during growth and division.

Physiological and electron microscopic measurements show that the periplasmic space of *E. coli* and *S. typhimurium* is approximately 20 to 40% of the total cell volume in normal growth media (110). Physiological measurements were done on cell suspensions by measuring the distribution of radioactive substances capable of penetrating both the inner and outer membranes (water), the outer membrane only (sucrose), or neither membrane (inulin). From such measurements cytoplasmic, periplasmic, and total cellular volumes were determined. The volume of the periplasmic space determined by this method is in agreement with a similar measurement made from electron micrographs of unstained, fixed cells.

Physiological studies have also established that the periplasmic volume and osmolarity respond to changes in the osmotic strength of the external medium. For example, high concentrations of solutes which can penetrate the outer but not the inner membrane increase the osmolarity and volume of the periplasm, with a concomitant shrinkage of the cytoplasmic compartment. Since the cytoplasmic membrane is in fact flexible and unable to support an osmotic gradient, the periplasm and cytoplasm are iso-osmolar. The osmotic strength of these two compartments is approximately 170 mosM for cells in water and increases to 300 mosM in M63 minimal medium (110). This range reflects primarily changes in cell volume and not the loss of intracellular solutes. Since M63 minimal medium is 145 mosM, there is normally an osmotic gradient between the periplasm and the external environment, and this osmotic pressure (3.5 atm [ca. 350 kPa]) is exerted against the peptidoglycan layer.

Biochemical studies indicate that the osmolarity of the periplasm may be regulated at least in part by the biosynthesis and export of so-called membrane-derived polysaccharides to the periplasmic space. These periplasmic polysaccharides contain 8 to 10 glucose units in a branched structure linked by $\beta(1\rightarrow2)$ and $\beta(1\rightarrow6)$ bonds and are multiply substituted with 1-phosphoglycerol residues derived from membrane phosphatidylglycerol, as well as with *O*-succinyl ester residues. This results in an average molecular weight of 2,200 to 2,600 and a net charge of −5. In one study it has been reported that cells can correct for external osmolar fluctuations by regulating the synthesis of these polymers and that they can account for up to 7% of the dry weight of the cell in media of low osmolarity (63). In another study, synthesis of membrane-derived polysaccharides also was found to be regulated by the osmolarity of the medium, but in the sense opposite to that found in the first study (25). This contradiction appears to be due to strain differences (25). Regulation of the synthesis of the membrane-derived polysaccharides requires de novo protein synthesis, but the details of this regulation remain to be determined. The location and identity of the osmosensor and its tie-in with the biosynthesis of these polymers need to be elucidated. A putative regulatory locus has recently been identified (25).

The presence of the cationic, membrane-derived polysaccharides in the periplasm creates a significant Donnan equilibrium across the outer membrane, resulting in an outer membrane potential. This potential can be measured by the unequal distributions of radioactive Na^+ and Cl^- ions across the outer membrane and amounts to approximately 30 mV in cells growing in M63 minimal medium (110). The physiological relevance of this outer membrane potential remains uncertain. However, it is clear that the Donnan equilibrium would create a particular ionic composition in the periplasm which could help to regulate the activities of this compartment as well as those of the two surrounding membranes.

PROTEIN CONTENT OF THE PERIPLASM

Periplasmic proteins are probably localized differentially in the periplasmic space, since they may peripherally associate with the inner membrane, the outer membrane, or the peptidoglycan layer or may freely diffuse within this compartment. Periplasmic proteins have been largely defined by methods that selectively release the contents of the periplasmic compartment from the rest of the cell. Obviously, such methods represent a compromise between conditions that are stringent enough to disrupt the peripheral associations of periplasmic proteins with cell envelope structures and those that are mild enough to prevent the release of integral membrane or cytoplasmic proteins. Thus, certain methods release only a subset of periplasmic proteins, while others release both periplasmic and certain nonperiplasmic proteins. Clearly, additional criteria are needed to sub-

TABLE 1. Proteins released by spheroplasting or osmotic shock

Protein	Reference(s)[a]
Binding proteins	
Arabinose[b]	90
Arginine specific[b]	101
Cystine and diaminopimelic acid[b]	18
Galactose-glucose[b,c]	77
Glutamate-aspartate[b]	121
Glutamine[b,c]	119, 122
Histidine[c]	46
Leucine specific[b]	88
Leucine-isoleucine-valine[b,c]	91, 122
Lysine-arginine-ornithine[b]	100
Maltose[b]	62
Phosphate[b]	72
Ribose[b,c]	1, 120
Sulfate[c]	89
Thiamine[b]	43
Vitamin B_{12}[b]	112
Xylose[b]	26
Scavenging enzymes	
Acid phosphatase (pH 2.5)	30
Acid phosphatase (pH 4.5)	
ADP-glucose hydrolase	
Alkaline phosphatase	
L-Asparaginase	
Carboxypeptidase II	
Cyclic phosphodiesterase (3'-nucleotidase)	
Endonuclease I	
Nicotinate phosphoribosyl transferase	
Polygalacturonic acid transeliminase[d]	
Polyphosphatase	
Sugar phosphate phosphohydrolase[d]	
UDP-glucose hydrolase (5'-nucleotidase)	
Detoxifying enzymes	
Alkylsulfohydrolase	
Aminoglycoside 3'-phosphotransferase II[d]	
β-Lactamase	60
Streptomycin adenylating enzyme[d]	
Other proteins	
Cytochrome *c*	
Hydrogenase	
Nitrite reductase	
Phosphoglucomutase	
Phosphoglucose isomerase	
Nonperiplasmic proteins (released by osmotic shock only)	
Aminopeptidase N[e]	
Cytidine deaminase	
Deoxyriboaldolase	
Deoxyribomutase	
Elongation factor Tu	
Purine deoxynucleoside phosphorylase	
Purine phosphoribosyl transferase	
Thymidine phosphorylase	
Uridine phosphorylase	

[a] See Table 1 of reference 12 if no reference is given.
[b] *E. coli* protein.
[c] *S. typhimurium* protein.
[d] Protein that is probably periplasmic, but release by spheroplasting has not been reported.
[e] Released from only one *E. coli* strain.

stantiate the periplasmic location of a given protein. These criteria have included (i) localization based on histochemical studies using electron microscopy, (ii) lack of crypticity (for enzymes) in intact cells, (iii) inhibition of activity (for enzymes) or selective labelling by reagents that do not permeate the inner membrane, and (iv) selective release by certain envelope mutants. Most of these criteria only substantiate an envelope location and are not useful in confirming a periplasmic location.

Two methods have been commonly employed to release periplasmic proteins: spheroplast formation (19, 73) and osmotic shock (79). Spheroplasts are usually prepared in a concentrated sucrose solution by treatment of cells with lysozyme-EDTA. Presumably, removal of the peptidoglycan layer allows periplasmic proteins to leach out through the outer membrane which has been breached by EDTA. Osmotic shock involves pretreatment of cells with a concentrated sucrose solution and EDTA followed by a rapid dilution into medium of low osmotic strength. Periplasmic proteins presumably are expelled during this procedure by the sudden expansion of the inner membrane against the cell wall. Centrifugation is then used to remove the cells from the resulting periplasmic fraction. The profiles of periplasmic proteins released by these two methods differ somewhat, since osmotic shock releases a number of proteins that are not released during spheroplast formation (Table 1). Furthermore, certain of these proteins are not periplasmic but probably are derived from the cytoplasmic membrane. Therefore, release during spheroplast formation appears to provide the better operational definition for periplasmic proteins. Recently, a third method for releasing periplasmic proteins has been described which involves treatment of a cell suspension with chloroform and subsequent dilution and removal of cells (3). In addition to these major methods, some of the periplasmic proteins can be released simply by washing cells with Tris-KCl, Tris-EDTA, or sucrose-Tris-EDTA (for references see Beacham [12]).

A list of the known proteins that are released during spheroplast formation and osmotic shock in *E. coli* is given in Table 1. The known periplasmic proteins can be divided into several groups, which include (i) binding proteins that function in the transport of small molecules and in chemotaxis, (ii) "scavenging" enzymes which break down complex molecules into simpler precursors or which function in cell wall biogenesis, (iii) detoxifying enzymes that inactivate toxic molecules, and (iv) other proteins that do not fall into these simple groups.

Periplasmic binding proteins represent a relatively homogenous group that have been more extensively studied than other periplasmic proteins. Binding proteins for sugars, amino acids, vitamins, and ions have been characterized. They are generally abundant and have high affinities for their respective substrates (K_m = 10^{-7} to 10^{-6} M). Binding proteins interact with their respective inner membrane permeases, thereby allowing for the translocation of small molecules across the inner membrane. Certain carbohydrate-binding proteins have a dual function since they also interact with inner membrane proteins that are signal transducers for chemotaxis (e.g., maltose-binding protein binds to the *tar* gene product transducer, and ribose-binding protein and galactose-binding protein

bind to the *tsr* gene product transducer [see chapter 49]). Several binding proteins have been crystallized, and their structures have been determined (i.e., arabinose [80, 93], galactose [95], maltose [94], and sulfate [92]). They appear to have similar structures, since the molecules are ellipsoidal with two distinct globular domains. At least for the arabinose- and sulfate-binding proteins, the substrate-binding site is located deep within a cleft formed by the close packing of these two domains. Further work is needed to determine whether the other binding proteins share these similarities.

ROLE OF THE SIGNAL PEPTIDE IN DIRECTING EXPORT

Periplasmic proteins, like other envelope proteins, are made as larger precursors containing an amino-terminal signal peptide of 20 to 40 amino acid residues which is cleaved during export. The amino acid sequence for signal peptides of 13 periplasmic proteins for *E. coli* is known. These proteins include alkaline phosphatase (50), eight binding proteins (arabinose and galactose [105], histidine and lysine-arginine-orithine [45], isoleucine-valine [69], leucine [88], maltose [13], and phosphate [72]), two β-lactamases (AmpC [60] and TEM [111]), and the two subunits of the heat-labile enterotoxin (27, 109). These signal peptides, like other procaryotic signal peptides, contain at least three conserved features: (i) the amino terminus has one or two positively charged amino acid residues; (ii) the amino terminus is followed by a stretch of 14 to 20 neutral, primarily hydrophobic amino acids known as the hydrophobic core; and (iii) a stretch of approximately six amino acid residues after the hydrophobic core is predicted to form a reverse turn. This segment ends in a consensus processing site denoted AXB, where B is the last amino acid residue of the signal peptide and is alanine, glycine, or serine and A includes these amino acids as well as leucine, valine, and isoleucine. It is not known if this sequence around the processing site is required for recognition by the signal peptidase or if the preference for small amino acid residues is merely for steric considerations. In addition, signal peptides are predicted to assume an α-helical or β-sheet secondary structure (4) according to the rules developed by Chou and Fasman (24). Such secondary structures have been corroborated using model synthetic peptides (102).

Since signal peptides from periplasmic proteins are structurally similar to their inner and outer membrane protein counterparts, it seems likely that the signal peptide plays no role in determining the ultimate location of a given protein. Rather, analysis of mutants in this sequence indicates that it serves to promote early steps in protein secretion. Signal sequence mutations that affect translation, secretion, and processing have been isolated and are caused by amino acid alterations in the basic amino terminus, the hydrophobic core, and the processing site, respectively. These alterations are shown in Fig. 1. Since similar results have been obtained with all envelope proteins, these data will be discussed collectively. For purposes of discussion, mutations in the signal sequence will be numbered according to the amino acid residue that is altered, starting with the amino terminus.

Basic Amino Terminus

The charge of the amino terminus of the signal peptide may be important in the coupling of translation to secretion. Mutants in which the amino terminus of the signal peptide of the major outer membrane lipoprotein (Lpp) is uncharged show nearly wild-type levels of Lpp synthesis and secretion (53, 116). However, mutants in which the amino terminus of the Lpp signal peptide has a net negative charge show a two- to fivefold reduction in Lpp synthesis and slower rates of Lpp secretion and processing (Fig. 1). A mutant in which the amino terminus of the lambda receptor (LamB) signal peptide is missing one of two basic amino acid residues is similarly reduced for LamB expression (Fig. 1) (44). It was possible to rule out an effect of mRNA secondary structure on LamB translation. This implies that the amino-terminal region of this signal peptide may also be involved in coupling of translation to secretion.

Hydrophobic Core Region

The hydrophobic core region of the signal peptide appears to be important in the association of the exported protein with the inner membrane and its translocation through the bilayer. Mutations in the hydrophobic core of the signal peptide can result in the accumulation of unsecreted protein precursors within the cytoplasm and cytoplasmic membrane of the cell. Three explanations have been offered for such defects, depending on the type of alteration present.

(i) Charged amino acids and deletions disrupt the required hydrophobicity of the core region, either in a site-specific way or by reducing the length below a certain minimal value needed for function (14). Most mutations that strongly block export of the TEM β-lactamase (Bla), LamB, maltose-binding protein (MalE), and alkaline phosphatase (PhoA) are of this type (Fig. 1). Suppressor analysis has been used to show that the length of the hydrophobic core region is critical in MalE signal peptide function. Mal^+ intragenic suppressor mutations have been isolated from strains that have charged amino acid substitutions or a deletion within the hydrophobic core region of this signal peptide. Most mutations that restore secretion of MalE lengthen the disrupted hydrophobic core region by insertion of additional hydrophobic amino acid residues or by extension of the amino-terminal boundary of the hydrophobic core by at least two amino acid residues (Fig. 2) (8; P. Bassford, personal communication).

(ii) Certain amino acid alterations destabilize a secondary structure necessary for signal peptide function. The presence of the helix-destabilizing amino acids proline and glycine in mutant signal peptides has been interpreted in these terms. The best documented example of a secondary structure defect is a small deletion in the LamB signal peptide that removes amino acid residues 10 through 13, which would have a net effect of destabilizing an α-helix by bringing the helix-destabilizing amino acids Pro-9

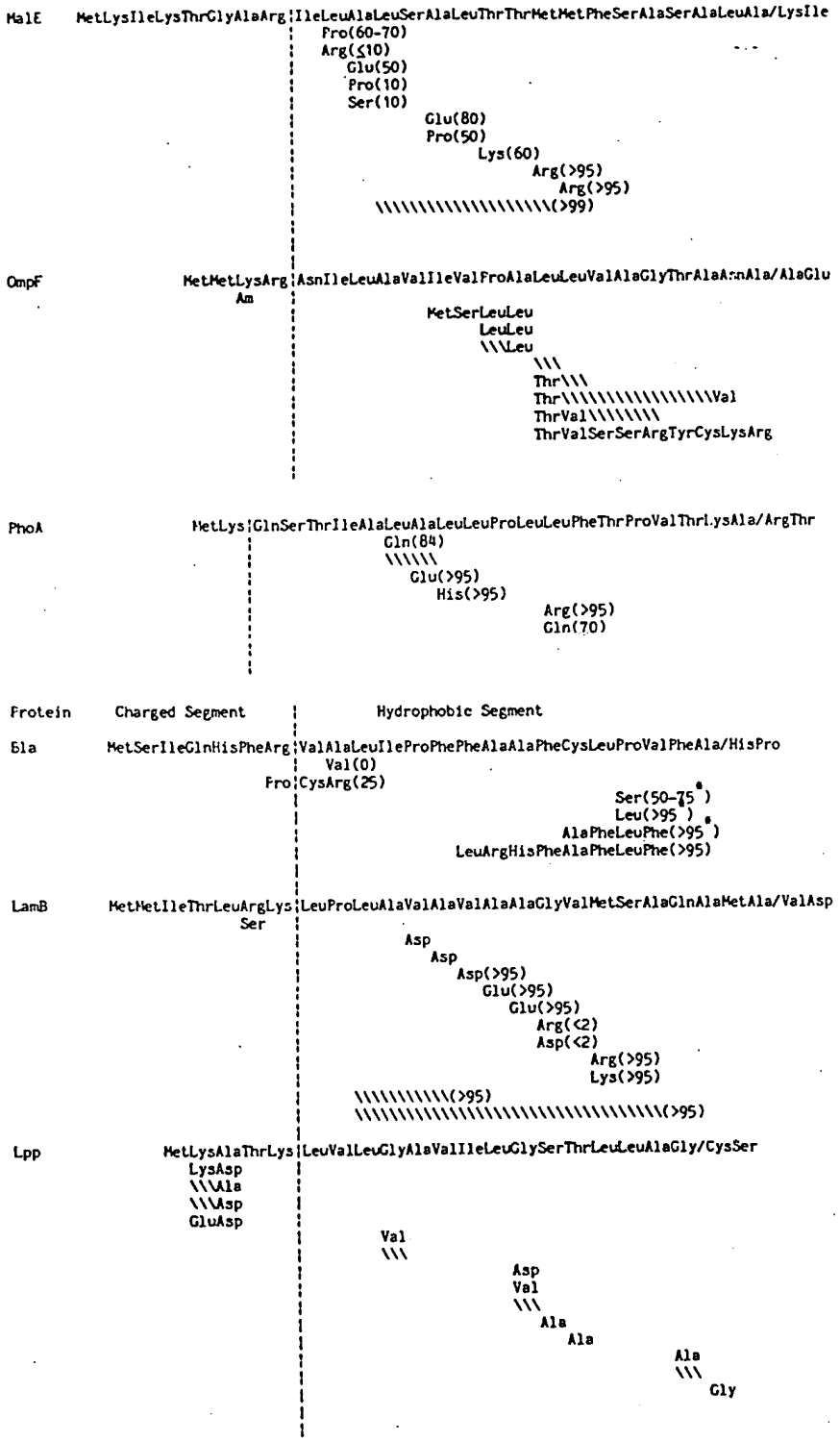

FIG. 1. *E. coli* signal sequence mutations. The amino acid(s) altered by a given mutation is indicated below the wild-type signal sequence. Deletions are indicated by hatching; Am indicates an amber mutation. Numbers in parentheses indicate the percentage of export or processing that is blocked by a given mutation; asterisks indicate a processing defect only. Slashes within sequences indicate the signal peptide processing site. References are as follows: Bla (66), LamB (38, 39, 44), Lpp (51–54, 71, 115, 116), MalE (8, 9, 13), OmpF (E. Sodergren, personal communication), PhoA (74; S. Michaelis, J. Hunt, and J. Beckwith, personal communication).

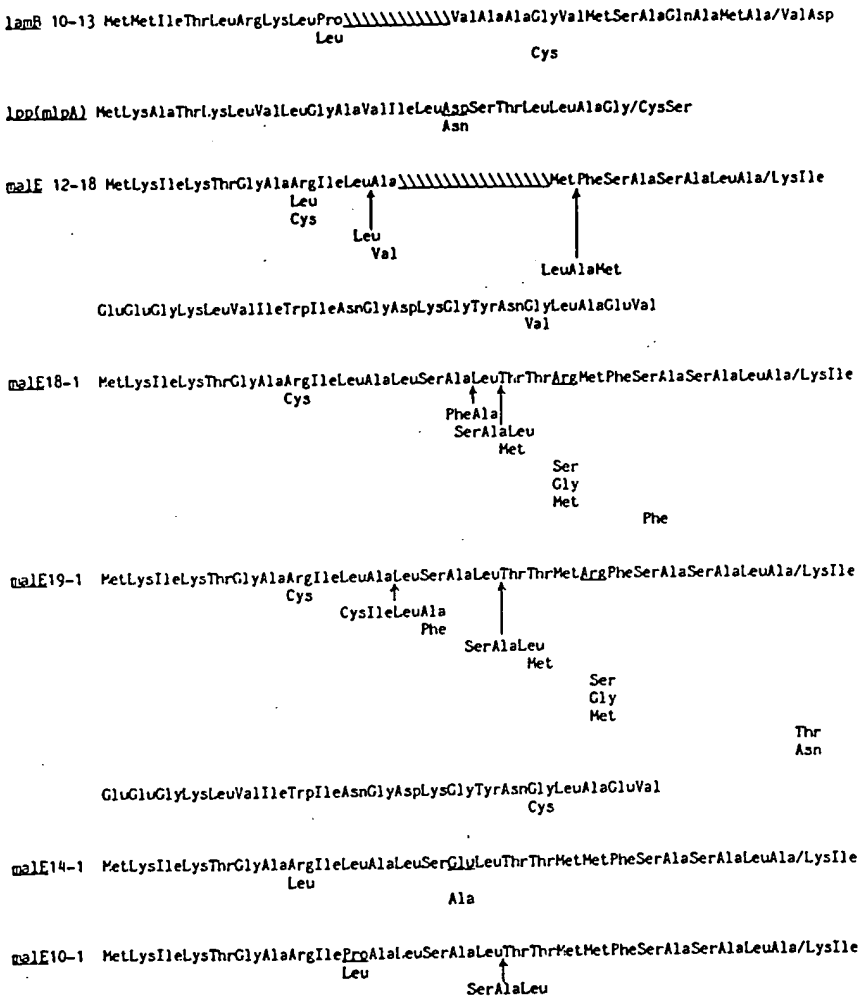

FIG. 2. Intragenic suppressors of signal sequence mutations. The underlined amino acids indicate the position of the primary mutation. Deletions are indicated by hatching. The amino acids altered by the second site suppressor mutations are given below the mutant signal sequence. Upward arrows indicate the insertion of additional amino acids into the signal sequence. Slashes indicate the signal sequence processing site. References are as follows: *lamB*10-13 (40), *lpp* (114), *malE* 12-18 (8), *malE*18-1, *malE*19-1, *malE*14-1, and *malE*10-1 (P. Bassford, personal communication).

and Gly-17 close together (Fig. 1). In support of this hypothesis, revertants of this deletion mutant that restore LamB export are found to have changed either of these two residues to an alternate amino acid (Fig. 2) (40).

(iii) Certain amino acid alterations have a positional effect because they disrupt a recognition site within the signal peptide that interacts with export machinery components. Since a biochemical system for studying this interaction in *E. coli* has not yet been perfected, this explanation remains speculative. However, there are numerous examples of positional effects of signal sequence mutations. For example, certain structurally similar and adjacent mutations within the MalE signal peptide (Arg-10 and Glu-11, Pro-10 and Pro-11 [Fig. 1]) block MalE export to very different extents. Clearly, further genetic and biochemical characterization of these mutants is required in order to determine the cause of such positional effects.

Processing Site

The region of the signal peptide around the processing site appears to play a role in processing only. Mutations in this region, either within the signal sequence or early in the mature sequence, can slow or eliminate processing, but they generally do not affect export of the mutant protein. The known processing mutations span a region around the cleavage site that includes the last four amino acid residues of the signal sequence and up to the first two amino acid residues of the mature sequence (66, 104). However, a systematic mutant analysis of the limits of this region has not been carried out. At least in certain cases such mutations directly affect the cleavage reaction of the precursor protein with the signal peptidase, as this can be demonstrated directly in vitro (104). Although export to the correct cellular compartment does not require processing, the maintenance of the uncleaved signal

-ptide can have dramatic effects on the solubility and topology of the secreted protein precursor (66).

ROLE OF MATURE PROTEIN SEQUENCES IN DIRECTING EXPORT

Since proteins destined for export to different locations have signal sequences of similar structure and function, it is likely that the more distal steps in export depend on sequences located in the mature portion of the secreted protein. Sequences that are important in the localization of proteins have been termed topogenic sequences. A gene fusion approach has been developed to locate the topogenic sequences present on the polypeptide chain of secreted proteins. By fusing the *lacZ* gene that encodes the normally cytoplasmic enzyme β-galactosidase to increasingly larger amounts of a gene coding for an exported protein, a nested set of hybrid proteins can be produced. These contain increased amounts of the amino-terminal portion of the secreted protein fused to a constant amount of enzymatically active β-galactosidase at the carboxy terminus (9). The intracellular distribution of the different-sized hybrid proteins can indicate where the localization information resides on the polypeptide chain of the secreted protein. This approach assumes that β-galactosidase is a passive carrier which does not positively or negatively affect the export information to which it is fused. As discussed below, this is not always the case.

The most extensive gene fusion analysis of a periplasmic protein has been done with MalE, which is expressed at high levels only in the presence of maltose. A *malE-lacZ* fusion encoding 14 amino acid residues of the MalE signal sequence fused to β-galactosidase does not initiate export and is found in the cytoplasm (13). In contrast, larger *malE-lacZ* fusions which encode an intact signal sequence and from 23 to 300 amino acid residues of mature MalE are exported out of the cytoplasm and are found in the inner membrane (98). Such secretion is abortive since the β-galactosidase portion of the hybrid protein apparently cannot pass through the membrane into the periplasm, but instead gets stuck in the secretion sites in the inner membrane (9). Consistent with this notion, synthesis of large amounts of such fusion proteins by the addition of maltose interferes with export and results in the cytoplasmic accumulation of precursors to many normally secreted proteins. In fact, in such maltose-sensitive strains the export of all of the major outer membrane proteins and most of the periplasmic proteins is blocked during induction, implying that envelope proteins probably share a common early step(s) in their secretion (56; L. Liss and D. Oliver, unpublished data). Since here β-galactosidase interferes with secretion, the gene fusion approach cannot be used to locate the export information contained on MalE or other periplasmic proteins.

An alternative approach to determine whether mature sequences are required for the secretion of periplasmic proteins is to study the secretion of truncated proteins produced by chain-terminating mutants. In one study, two *malE* amber mutants were used which made proteins that were 30 and 90% of the size of wild-type malE protein. Although both proteins were processed, only the larger one was found in the periplasm (57). However, protease protection experiments were used to show that at least a portion of the smaller amber fragment was exterior to the cytoplasmic membrane. Whether the additional sequences present on the larger protein are needed for completion of traversal through or release from the inner membrane or whether they allow the larger protein to assume a water-soluble conformation in the periplasm is unclear. Similar studies have been done with chain-terminating mutants in the *bla* gene (65) and in the gene encoding arginine-binding protein (22). Taken together, these studies indicate that certain carboxy-terminal sequences are not required for export to the periplasmic space.

Three lines of evidence support the idea that export-specific information is located within the mature portion of MalE protein. However, this evidence is indirect and needs more direct confirmation.

(i) MalE signal sequence mutations in the hydrophobic core can be weakly suppressed by amino acid alterations in the mature region (Fig. 2) (8). However, the suppressor mutations are sufficiently close to the signal peptide that they may restore correct folding without normally facilitating the export of wild-type MalE.

(ii) The synthesis of secreted proteins with signal sequence defects can interfere with the export of normal envelope proteins, causing a delay in their export and processing (6). Interference requires not only a mutation in the signal sequence of the interfering protein, but also the presence of certain carboxy-terminal sequences (V. Bankaitis and P. Bassford, personal communication).

(iii) Mature sequences within MalE determine the rate at which MalE-LacZ hybrid proteins are abortively exported and processed (98). For example, the MalE portion of a shorter hybrid protein (signal sequence plus 23 amino acid residues of mature MalE) is secreted into the membrane and processed posttranslationally with slow kinetics. On the other hand, very rapid kinetics, similar to those in wild-type MalE export, are seen with a longer hybrid protein (signal sequence plus 189 amino acid residues of mature MalE). However, the proximity of β-galactosidase sequences to the signal sequence could also explain these results.

Since the presence of export-specific information within the mature portion of a periplasmic protein has not yet been proven, it is possible that this region is largely devoid of such information. In contrast, topogenic sequences have been identified in the mature portion of inner and outer membrane proteins. Certain proteins that span the inner membrane possess a stop-transfer or membrane-anchoring sequence made up of 19 to 23 uncharged, primarily hydrophobic amino acid residues flanked by charged amino acid residues (32). This sequence apparently functions to stop export of the protein through the membrane as well as to serve as a membrane anchor, since deletion of this sequence results in a periplasmic location of the truncated protein. Topogenic sequences have also been identified in the mature portion of LamB; the sequences are required to target this protein to the outer membrane. There is a sequence within the first 49 mature amino acid residues of LamB which allows this protein to achieve an outer membrane location

(15). A second sequence further in LamB appears to improve the efficiency of export (17).

One problem with defining a given topogenic sequence is the difficulty in clearly discriminating whether the sequence is required for export to a given compartment or for the thermodynamic stability of the protein once it has been properly exported. Thus, even if a mutant protein is correctly secreted, it may be unable to associate stably with the correct cellular compartment. However, the studies cited above tend to indicate that periplasmic proteins may be devoid of additional topogenic signals within their mature sequence. This would allow them to completely traverse the inner membrane in an uninterrupted fashion and be released into the periplasmic space. Clearly this interpretation is speculative, and further work is needed to resolve this point.

PHYSIOLOGY OF PROTEIN EXPORT

The timing of protein secretion with respect to translation has been the subject of a number of studies. These studies were aimed at understanding whether protein synthesis plays a role in protein secretion and determining whether the translocation of the polypeptide chain through the inner membrane occurs in a conformationally extended or folded form. It has been demonstrated that a number of periplasmic and outer membrane proteins are preferentially synthesized on membrane-bound polysomes (96). Direct proof of cotranslational secretion of PhoA has been obtained by labeling spheroplasts with a membrane-impermeable reagent and subsequently completing the synthesis of these externally labeled, nascent chains in vitro (108). In contrast, the synthesis of Bla protein is complete before translocation and processing are detected (65). Therefore, it appears that secreted proteins fall into at least two categories, depending on whether their synthesis is complete before their export and processing commences. It has been possible to study such kinetics in detail by using a gel system that determines the proportion of nascent chains that have crossed the inner membrane enough either to be sensitive to externally added protease or to have had their signal peptide processed. As expected, certain proteins are processed completely cotranslationally (AmpC), while others are processed completely posttranslationally (Bla), but most show a mixture of both modes of processing (arabinose-binding protein, MalE, PhoA, LamB, and OmpA) (61). Of interest is that all proteins examined needed to reach 80% of their full length before any traversal or processing was detected (97). Although these studies do not rule out the possibility that polypeptide chain traversal occurs in an extended rather than in a folded form, they do indicate that traversal of even a nascent protein chain occurs in a nonlinear fashion, seemingly independent of chain elongation.

Studies on the energetics of protein secretion indicate than an energized membrane or proton motive force is required for proteins to cross the cytoplasmic membrane. Either the membrane potential or the pH gradient appears to be sufficient for this requirement (5). Reagents such as the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone or the potassium ionophore valinomycin at concentrations that reduce the membrane proton motive force also block secretion of a large number of envelope proteins in a reversible manner (28, 41, 124). This export block does not appear to be due to a reduction in the intracellular ATP levels. It remains unknown whether the proton motive force plays a direct role in protein export (for example, as an energy source) or an indirect role (for example, in maintaining membrane structure).

EXPORT MACHINERY

The existence of a complex export machinery that is required for the secretion of many envelope proteins is clearly suggested by both biochemical and genetic studies. Progress with the in vitro secretion system for *E. coli* has been slow compared with its mammalian counterpart due to difficulty in preparing protein-synthesizing extracts and membrane vesicles that are active in protein translocation. On the other hand, progress in genetically defining the components required for protein secretion has been rapid, yet we are unsure as to the precise function that these components serve in this process. Clearly, future progress in this area will require an interplay between these two different approaches.

Biochemical Studies

In vitro protein secretion systems have been developed which combine in vitro protein synthesis with secretion into inverted vesicles prepared from membranes. Authentic secretion and processing of Bla, OmpA, and PhoA have been demonstrated by several criteria (23, 75, 99). These systems mimic the in vivo situation in their sensitivity to agents that reduce the membrane proton motive force or which perturb membrane structure (ethanol and phenethyl alcohol). Posttranslational secretion takes place at high efficiency in these systems, although the rate of translocation is extremely slow (approximately 15 min) compared with normal in vivo rates, making it uncertain whether the normal and complete export pathway is being utilized. Export has been shown to require a soluble factor sedimenting at about 12S (76), as well as a protein factor(s) localized on the membranes (23). Further characterization of these and other factors is needed.

Certain secreted proteins apparently do not require export machinery components during their biogenesis. A prime example is the trans-inner membrane coat protein of bacteriophage M13. It has been shown that radiochemically pure coat protein precursor integrates into liposomes containing only *E. coli* phospholipids and the highly purified processing enzyme, that the precursor is correctly processed, and that up to 70% of the mature protein correctly spans the membrane (81). These in vitro experiments coincide with in vivo findings that coat protein is secreted posttranslationally (59) and is not blocked in mutants showing general secretion defects (see below) (123). Thus, certain proteins apparently completely catalyze their own export, although this is probably not the general case for most envelope proteins in *E. coli*.

Two distinct signal-peptide-processing enzymes have been identified and purified from *E. coli* inner membranes. Their genes have been mapped, cloned, and sequenced. Signal peptidase I, or leader pepti-

TABLE 2. Genetic loci implicated in protein secretion

Gene	Min	Wild-type or mutant phenotype(s)[a]	Reference(s)
expA	22	Ts on minimal media; decreases amount of certain envelope proteins	29
lepB	55.5	Gene for signal peptidase I; essential gene	124, 125
lspA	0.5	Gene for signal peptidase II; essential gene; Ts mutant available	49, 127, 128
perA (envZ)	75	Decreases amount of certain envelope proteins; transcriptional regulation of OmpC and OmpF	117
prlA (secY)	72	Ts allele shows general export defect; other alleles suppress signal sequence mutations or *secA*(Ts) mutants	21, 37, 107
prlB (rbsB)	84	Deletion in ribose-binding protein; suppresses *lamB* signal sequence mutations without processing	16, 36, 37
prlC	68	Suppressor of *lamB* and *malE* signal sequence mutations	16, 36, 37
prlD	2.5	Suppressor of certain *malE* and *lamB* signal sequence mutations; Synergistic export defects with certain *prlA* alleles	7
prlE	8.5	Slight Cs phenotype; reduces export of MalE, PhoA, and LamB; probably equivalent to *secD* and *ssaD*	See text
prlF	70	Relieves maltose sensitivity of *lamB-lacZ* and *malE-lacZ* fusions; slight Cs phenotype	64
secA	2.5	Ts alleles block export of most envelope proteins; Am allele blocks translation of MalE	84, 85
secB	81	Tn*5* insertions (nonessential gene); reduces export of MalE, LamB, and OmpF but not PhoA and RbsB	67
secC (rpsO)	69	Gene for ribosome protein S15; Cs alleles suppress *secA*(Ts) mutants; translational block of MalE, RbsB, LamB, and OmpF	42
ssaD	10	Cs allele suppresses *secA*(Ts) mutant; decreased expression of MalE; probably equivalent to *secD* and *prlE*	82
ssaE	50	Cs allele suppresses *secA*(Ts) mutant; decreased expression of MalE	82
ssaF	83	Cs allele suppresses *secA*(Ts) mutant; decreased expression of MalE; probably *rpmH* coding for ribosomal protein L34	82
ssaG	41	Cs allele suppresses *secA*(Ts) mutant; decreased expression of MalE	82
ssaH	94.5	Cs allele suppresses *secA*(Ts) mutant; decreased expression of MalE	82
ssyA	54	Cs allele suppresses *secY*(Ts) mutant; slower protein synthesis	106
ssyB	10	Cs allele suppresses *secY*(Ts) mutant	See text
ssyC	69	Cs allele suppresses *secY*(Ts) mutant	See text
ssyD	3	Cs allele suppresses *secY*(Ts) mutant	See text
ssyE	72	Cs allele suppresses *secY*(Ts) mutant	See text
ssyF	20	Cs allele suppresses *secY*(Ts) mutant	See text

[a] Am, Amber; Cs, cold sensitive; Ts, temperature sensitive.

dase, appears to be the general enzyme for processing precursors to periplasmic as well as inner and outer membrane proteins (leucine- and isoleucine-valine-binding proteins, MalE, LamB, OmpA, OmpF, and M13 coat protein) (118). The purified enzyme consists of a single polypeptide chain of 36,000 daltons and is found in both the inner and outer membranes (129). The enzyme is anchored in the inner membrane by a short amino-terminal tail and contains a large periplasmic region (125). Thus, it is itself a secreted protein, although it does not possess a cleaved signal peptide. However, its secretion is blocked by agents which reduce the membrane potential as well as in mutants showing general secretion defects (see below) (123, 124). No mutations in this enzyme have yet been reported, although the gene appears to be essential for cell survival (31).

Signal peptidase II, or lipoprotein-specific signal peptidase, is the processing enzyme for glyceride-modified lipoprotein precursors. Using an inhibitor of this enzyme, the antibiotic globomycin, or a mutant in the structural gene for the enzyme, it has been possible to show that signal peptidase II is responsible for the processing of the Braun lipoprotein as well as seven additional minor lipoproteins found in the cell envelope (126, 127). The purified enzyme consists of a single polypeptide chain of 18,000 daltons and is a highly hydrophobic protein found exclusively in the inner membrane (49, 113, 128). In keeping with its specificity, it only processes the glyceride-modified form of lipoprotein precursors.

In addition to signal peptidases, the cell must possess an enzyme(s) that degrades the processed signal peptide. The inner membrane enzyme, protease IV, has been shown to be responsible for the degradation of the Lpp signal peptide, but only after its cleavage by signal peptidase II (48). Whether this is the only enzyme that hydrolyzes signal peptides in *E. coli* remains to be determined.

Genetic Studies

Two general types of genetic selections have been successfully applied to the isolation of mutations in the cellular export machinery. The first selection relies on the existence of a mutation within the signal sequence of a secreted protein that blocks its export and therefore its function. By selecting for restoration of function, it has been possible to isolate extragenic suppressor mutants which compensate for the original signal sequence defect apparently by altering export machinery components. The *prlA*, *prlB*, *prlC*, and *prlD* mutations have been isolated in this fashion (Table 2) (7, 37). The second selection relies on the fact that hybrid proteins containing a carboxy-terminal β-galactosidase moiety have a Lac$^-$ phenotype when

they are exported to the inner or outer membrane. By selecting for Lac$^+$ derivatives, it has been possible to isolate extragenic suppressor mutants which apparently partially inactivate the export machinery so that the hybrid protein is not exported from the cytoplasm and remains enzymatically active. The *secA*, *secB*, *secD*, and *prlE* mutations have been isolated in this fashion (67, 84; C. Cardel, J. Hunt, S. Michaelis, and J. Beckwith, personal communication; S. Benson, D. Kiino, and C. Cardel, personal communication).

It has also been possible to use some of these secretion mutants as a starting point for defining additional genes involved in the export process by isolating extragenic suppressor mutations of conditional lethal secretion mutants. Suppressors of temperature-sensitive mutants in *secA* and *secY* have been isolated using this approach (Table 2) (82, 106).

By using these genetic methods, a large number of loci have been defined. They are listed in Table 2. The data indicating that these loci are directly involved in protein secretion are strong in certain cases and weak in others. Certain loci, in fact, do not appear to be directly involved in protein secretion (e.g., *prlB* [Table 2]). In general, physiological criteria have been used to infer that a given mutation directly affects protein secretion. Secretion mutants have been inferred when the following defects have been found: (i) accumulation of protein precursors, (ii) restored secretion of an envelope protein containing a defective signal peptide, (iii) specific reduction in the synthesis of several exported proteins under different regulatory controls, and (iv) reduction in the synthesis of an exported protein, but normal synthesis of that protein when it contains a signal sequence mutation. Clearly, these physiological defects could also be due to indirect effects, and biochemical characterization of the function of these gene products is sorely needed. Nonetheless, there has been extensive characterization of certain secretion mutants. This has allowed important inferences to be drawn about the general requirement for the export machinery in envelope protein biogenesis and its potential tie-in with the translation machinery. These data are discussed below.

***prlA* and *prlD* mutants.** The *prlA* and *prlD* mutants were isolated as extragenic suppressors of signal sequence mutations in particular envelope proteins. This suppression is more general, since both mutants have been shown to suppress signal sequence mutations in the hydrophobic core of a variety of envelope proteins (LamB, MalE, PhoA, and OmpF for *prlA* [36]; LamB and MalE for *prlD* [7]), resulting in the secretion and processing of the normally export-defective protein to the correct cellular location. The *prlA* mutants show a range in suppression efficiencies, depending on the signal sequence allele, but in general are strong suppressors of signal sequence defects. In contrast, the single *prlD* mutant isolated is a weak suppressor of certain signal sequence defects only and can, in fact, exacerbate such defects. Such allele specificity has been interpreted as implying that these two proteins interact directly with the signal peptide. Although neither mutant alone shows export defects with normal envelope proteins, certain *prlA prlD* double mutants show severe secretion defects which result in the accumulation of precursors of periplasmic and outer membrane proteins (MalE, RbsB, LamB, and OmpA [7]).

A conditional lethal temperature-sensitive mutant in the *prlA* gene has been isolated using localized mutagenesis (107). When this mutant is shifted to the nonpermissive temperature, it accumulates precursors to a number of exported proteins in the cytoplasm. These proteins include MalE, OmpA, OmpF, and Lpp. In a different study it has been shown that reducing the level of wild-type PrlA also results in a similar export-defective phenotype (58). Since both export-defective and signal sequence suppressor mutations have been obtained in *prlA*, the evidence that this gene is directly involved in protein export is compelling. Biochemical characterization of the function of the recently identified PrlA protein (55) should help to clarify the role of the protein in secretion and its potential interaction with PrlD and the signal peptide.

***secA* and *ssa* mutants.** Temperature-sensitive mutants in the *secA* gene were isolated using a genetic selection that demands a partial defect in export at the permissive temperature (84). Growth at the nonpermissive temperature results in a complete block of secretion, with the cytoplasmic accumulation of a number of envelope proteins. A more thorough analysis of a *secA* mutant under these conditions showed that export is blocked for all of the major outer membrane proteins and for most, but not all, of the periplasmic proteins (L. Liss and D. Oliver, unpublished data). In addition, under these conditions export of the transmembrane protein, signal peptidase I, is also blocked, while M13 coat protein biogenesis is not (123). Thus, SecA appears to be an essential component of the major secretion system in *E. coli* which functions in an early step in periplasmic, inner, and outer membrane protein biogenesis. This does not preclude other, minor export pathways that do not utilize SecA.

In order to identify additional components of the export machinery which interact with SecA, extragenic suppressors of a *secA*(Ts) mutant have been isolated. This analysis has been confined to essential genes by isolating suppressors which not only allow growth at the high temperature, but also render the cell cold sensitive for growth at a low temperature. These suppressors fall into at least seven genes (Table 2). Some of these suppressors may be directly involved in protein export since they (i) restore secretion in the *secA*(Ts) mutant at the high temperature, (ii) still require normal SecA levels, and (iii) often show export-related defects as cold-sensitive single mutants.

One of the suppressors falls into the *prlA* gene, which was previously implicated in protein export (21). This suggests that PrlA and SecA probably are components of a common export machinery. This conclusion is further supported by the fact that *prlA*-mediated suppression of signal sequence mutations depends on the presence of normal SecA levels in the cell (86). Two other suppressors are in ribosomal genes, indicating that ribosomes may interact with the export machinery during secretion. In this category are *secC* (*rpsO* which codes for S15) (42) and *ssaF* (*rpmH* which codes for L34) (82). Not only do these mutants suppress the *secA*(Ts) export defect at the high temperature, but also, as single cold-sensitive mutants, they show synthetic defects for exported proteins but not for cytoplasmic proteins at the low tem-

perature. One interpretation of these results is that certain portions of the ribosome interact with the export machinery to couple protein synthesis with secretion. Whether this or another explanation is correct will require additional genetic and biochemical studies.

CONCLUSION

A preliminary understanding of the structure and contents of the periplasmic space has been obtained. Areas for further research include (i) identification of additional periplasmic proteins which are not detected by simple enzymatic and binding assays (some of these proteins may play important roles in cell envelope biogenesis and cell division; (ii) clarification of the extent to which various periplasmic proteins are confined to particular microenvironments within this region and whether this serves to regulate their activities; (iii) clarification of the physiology of membrane-derived polysaccharide synthesis, its mechanism of regulation, and its role in conditioning the periplasmic environment; and (iv) elucidation of the different forms of communication between the periplasm and the cytoplasm which help to regulate the different activities in these two compartments.

Considerable progress has been made in understanding the biogenesis of periplasmic proteins and secreted proteins in general. Areas of progress include (i) elucidation of the essential nature of the signal sequence and a preliminary understanding of some of the structural features necessary for signal peptide function; (ii) definition of certain topogenic sequences present in the mature portion of inner and outer membrane proteins that are required in more distal steps in protein secretion; (iii) identification of two major signal peptidases that process different envelope proteins; and (iv) identification via mutant analysis of a number of genes affecting protein secretion, at least some of which should define components of a general protein export pathway in *E. coli*. Further research will be needed in order to dissect biochemically the various steps in export that are mediated by the signal sequence and other topogenic sequences and to elucidate the role of the various components of the export machinery in executing these steps. An interplay between the existing genetic and biochemical approaches should allow rapid progress in this field.

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